FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 514274-2001
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U S APPLICATION NO. (If known see 37 C.F R 1.5) 09/868760
INTERNATIONAL APPLICATION NO. PCT/NZ99/00227	INTERNATIONAL FILING DATE 23 DECEMBER 1999	PRIORITY DATE CLAIMED 23 DECEMBER 1998

TITLE OF INVENTION **SERINE PROTEASE INHIBITORS**

APPLICANT(S) FOR DO/EO/US **Paul Douglas SCOTTI, Sally Caroline DEARING, David Roger GREENWOOD, Richard David NEWCOMB**

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 – 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

PCT/RO/101, PCT/ISA/210, PCT/IPEA/401, 408,409, 416, PTO Form 1449, references for IDS, 4 sheets of drawings

EXPRESS MAIL

Mailing Label Number: **EL742691527US**

Date of Deposit: **June 21, 2001**

I hereby certify that this paper or fee is being deposited with the United States Postal Service

"Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents and Trademarks, Box PCT Washington, DC 20231.

Charles Jackson
(Typed or printed name of person mailing paper or fee)

Charles Jackson
(Signature of person mailing paper or fee)

531 Rec'd PCT 21 JUN 2001

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.50)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NO.
09/868760	PCT/NZ99/00227	514274-2001

21. ☒ The following fees are submitted	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) Nor international search fee (37CFR 1.445(a)(2) paid to USPTO And International Search Report not prepared by the EPO or JPO .....$1000 00 International preliminary examination fee (37 C.F.R. 1 482) not paid to USPTO but International Search Report prepared by the EPO or JPO .....$860.00 International preliminary examination fee (37 CFR 1 482) not paid to USPTO but International search fee (37 CFR 1 445(a)(2)) paid to USPTO .....$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) But all claims did not satisfy provisions of PCT Article 33(1)-(4 ..... $690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) And all claims satisfied provisions of PCT Article 33(1)-(4).....$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 Months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total Claims	24 - 20 =	4	x **$18.00**	**$ 72.00**	
Independent Claims	4 - 3 =	1	x **$80.00**	**$ 80.00**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
			TOTAL OF ABOVE CALCULATIONS =	**$ 1,012.00**	
☐ Applicant claims small entity status. See 37 C.F.R. 1.27. The fees indicated above are reduced by ½. +				$	
			SUBTOTAL =	**$ 1,012.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 Months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
			TOTAL NATIONAL FEE =	**$ 1,012.00**	
Fee for recording the enclosed assignments (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
			TOTAL FEES ENCLOSED =	**$ 1,012.00**	
			Amount to be refunded:	$	
			Charged:	$	

a. ☒ A check in the amount of $**1,012.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0320**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. WARNING: Information on this form may become public. **Credit card information should not be included on this form**. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

William F. Lawrence, Esq.
FROMMER LAWRENCE & HAUG LLP
745 Fifth Avenue
New York, NY 10151

Date: June 21, 2001

SIGNATURE

William F. Lawrence, Esq.
NAME

28,029
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	SCOTTI, et al.
U.S. Serial No.	:	09/868,760
Filing Date	:	June 21, 2001
For	:	SERINE PROTEASE INHIBITORS
Examiner	:	To be Assigned
		745 Fifth Avenue, New York, NY 10151

EXPRESS MAIL

Mailing Label Number: EL 819163957 US

Date of Deposit: July 11, 2001

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Charles Jackson

(Typed or printed name of person mailing paper or fee)

Charles Jackson

(Signature of person mailing paper or fee)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Box Sequence
Washington, D.C. 20231

Dear Sir:

Applicants respectfully request acceptance of the enclosed paper copy and computer readable form of the Sequence Listing. It is also respectfully requested that the application be amended as follows:

IN THE SPECIFICATION:

Please amend the specification as follows:

Please replace the paragraph beginning at page 17, line 23, with the following rewritten paragraph

--The sequence data was then compared with amino acid sequences in searchable computer databases. Some sequences were found to be of particular interest:

a) a 10 amino acid residue sequence from he N-terminus of pernin (sequence (a) above showed only homology with an 8- base anti-thrombin protein sequence (SEQ ID NO: 9) from the terrestrial leeches (data from US patent 5,455,181 Oct 3, 1995: sequence 10).--;

Please replace the paragraph beginning at page 20, line 5, with the following rewritten paragraph:

--A suite of non-specific primers called pUZ5 was synthesized by Gibco-BRL for the initial sequencing based on the N-terminal sequence of pernin. The general formula was GAY GGN GAR CAR TGY AAY GAY GGN CAR AA (SEQ ID NO: 10) Where Y represents a pyrimidine base, R represents a purine base and N represents any one of the four-nucleotide bases. Sequencing was done, initially using pUZ5 and an oligo-dT based "bottom strand" primer from PCR amplified cDNA. Sequencing was done by dye-termination cycle sequencing using "BigDye" prism technology (Applied Biosystems Incorporated, USA) according to their instructions. Products were resolved on an ABI 377 automated sequencer. Following the initial sequencing of approximately 500 base pairs pernin-specific primers were constructed and used to complete the sequencing of the pernin gene.--

Immediately after page 23 and before the first page of claims (page24), if appropriate, please insert the enclosed pages identified as --Sequence Listing--. Please renumber the pages accordingly.

REMARKS

It is respectfully asserted that the sequence disclosure contained in the application now fully complies with the requirements set forth in 37 C.F.R. § 1.821 to § 1.825. Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with markings to show changes made."**

These amendments are introduced merely to assign the correct SEQ ID NO: and to place the nucleotide sequence listing in the application, (after the specification and before the claims). It is respectfully asserted that these amendments do not add any new matter.

In view of the amendments, remarks and enclosures, the application complies with the requirements for computer readable disclosure of the biological sequences under 37 C.F.R. §1.821-1.825. This response is being submitted without a formal Notice to Comply.

If any additional fees are incurred for entry and consideration of this Amendment, the Examiner is authorized to charge any fees or credit any overpayment to Deposit Account No. 50-0320.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP

By: Susan Lehnhardt

Susan K. Lehnhardt
Reg. No. 33,943
(212) 588-0800

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

Paragraph beginning at page 17, line 23 has been amended as follows:

The sequence data was then compared with amino acid sequences in searchable computer databases. Some sequences were found to be of particular interest:

a) a 10 amino acid residue sequence from he N-terminus of pernin (sequence (a) above showed only homology with an 8- base anti-thrombin protein sequence (SEQ ID NO: 9) from the terrestrial leeches (data from US patent 5,455,181 Oct 3, 1995: sequence 10).

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GAY GGN GAR CAR TGY AAY GAY GGN CAR AA (SEQ ID NO: 10)

Where Y represents a pyrimidine base, R represents a purine base and N represents any one of the four-nucleotide bases. Sequencing was done, initially using pUZ5 and an oligo-dT based "bottom strand" primer from PCR amplified cDNA. Sequencing was done by dye-termination cycle sequencing using "BigDye" prism technology (Applied Biosystems Incorporated, USA) according to their instructions. Products were resolved on an ABI 377 automated sequencer. Following the initial sequencing of approximately 500 base pairs pernin-specific primers were constructed and used to complete the sequencing of the pernin gene.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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U.S. Serial No.	:	09/868,760
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Charles D. Jackson
(Typed or printed name of person mailing paper or fee)

Charles D. Jackson
(Signature of person mailing paper or fee)

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 CFR §§ 1.821-1.825

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

The undersigned hereby states that the content of the printed Sequence Listing for the above-referenced application filed, and the computer readable copy, submitted in accordance with 37 CFR §§ 1.821(c) and (e), are the same and do not add any new matter.

In the unlikely event that the Patent Office determines that an extension and/or other relief is required as a result of this statement, applicants petition for any required relief including extensions of time and authorize the Assistant Commission to charge the cost of such petitions and/or other fees due to our **Deposit Account No.: 50-0320**.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP
Attorneys for Applicant

Susan Lehnhardt

Susan K. Lehnhardt
Reg. No. 33,943
(212) 588-0800

SEQUENCE LISTING

<110> The Horticulture and Food Research Institute of New Zealand Limited

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<130> 514274-2001

<150> PCT/NZ99/00227
<151> 1999-12-23

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gaaccaaacc ctcatatggc tagcagcctt caccaccatg tccatggcag catagagttg 180

tcacagaagg gtcatggagc tgtttatcta gaacttcatc ttgtcggatt caacacaagt 240

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gtgtcctacc atttagaggg atttaatgta agtgatgacc acaaagatca tctccatgac 780

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catggctgtc actccttagg cagaatgtac catggtcatg atgatgctca tgaccccaaa 1320

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35 40 45

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85 90 95

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Glu Leu Gly Asp Leu Gly Asp Ile His Asp Asp Asp His Gly Val Val
    290                 295                 300

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Val Leu Gly Arg Ser Ile Ala Ile His Gln Arg Asp His Leu His Lys
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            340                 345                 350

Glu Ile Val His Arg Ala Lys Cys Val Val Arg Pro Asn Thr Glu Ser
        355                 360                 365

Thr Gly Leu His His His Val Ser Gly Ser Ile Thr Phe Glu Gln Thr
    370                 375                 380

Pro Gly Gly Ser Thr His Met Thr Ala Asp Leu Lys Gly Phe Asn Val
385                 390                 395                 400

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                405                 410                 415

Gly Asp Met Ser His Gly Cys His Ser Leu Gly Arg Met Tyr His Gly
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Ile Asp Asp Ser His Gly Ile Val His Ser Thr Arg Thr Phe Asp His
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gaaccaaacc ctcatatggc tagcagcctt caccaccatg tccatggcag catagagttg 180

tcacagaagg gtcatggagc tgtttatcta gaacttcatc ttgtcggatt caacacaagt 240

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'y' is a pyrimidine nucleotide and 'r' is a pu ine nucleotide;

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SERINE PROTEASE INHIBITOR

This invention relates to a protein and compositions which contain it. More particularly, it relates to a protein which *inter alia* exhibits activity as a metal cation binding agent and as an anti-thrombin agent.

BACKGROUND

Thrombin is a serine protease involved in blood coagulation. It has specificity for the cleavage of arginine-lysine bonds as well as cleaving an arginine-threonine bond in pro-thrombin, releasing pre-thrombin which is subsequently cleaved to produce active thrombin. This active thrombin can then release more thrombin from pro-thrombin. In blood clotting and coagulation, thrombin cleaves fibrinopeptide B from fibrinogen as well as converting blood factors IX to IXa, V to Va, VIII to VIIIa and XIII to XIIIa.

Inhibitors of thrombin therefore inhibit coagulation and have application in any procedure where coagulation is undesirable. One such application is in the collection and storage of blood products. Another is in medicaments for preventing or reducing coagulation for example in treating or preventing cardiac malfunctions.

Anti-thrombin agents are known. One example is anti-thrombin III (AT-III). However, AT-III is capable of effectively inhibiting thrombin only in the presence of heparin.

The applicants have now identified a novel protein which has a range of activities, including anti-thrombin activity, and which when active against thrombin does not require heparin as a cofactor. It is towards this protein that the present invention is broadly directed.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides an isolated protein which has a molecular weight of about 55 kDa and an amino acid sequence which includes one or more of the following:

(a) DGEQCNDGQN (SEQ ID NO. 1)

(b) QGGHEVESERVACCVIGRA (SEQ ID NO. 2)

(c) GQSHPEIVH (SEQ ID NO. 3)

(d) YHGHDDA (SEQ ID NO. 4)

(e) VVNEVHH (SEQ ID NO. 5),

or an active fragment thereof.

In a further aspect, the invention provides an isolated protein which comprises the amino acid sequence of

D G E Q C N D G Q N K D D H H D D H H D D H H D D H D D D D
E T M H Y A Q C E M E P N P H M A S S L H H H V H G S I E L
S Q K G H G A V Y L E L H L V G F N T S E D H D D H H H G L
H L H M L G D M S A G C D S I G E L Y N A H P E K H A D P G
D L G D L V D D D R G V V N E V H H Y A W L D I D G T A P N
T E A L I G H S M T I L Q G S H T D A D T P A S R I A C C V
I G H G K A R P E T A A A L H H E L E E D K T E H Y A H C D
V R S N T H Q P K A L H H H V H G T I D F K Q V G Y G D L E
V S Y H L E G F N V S D D H K D H L H D V Q I Y A N G D L T
S G C D N L G A K Y D P H E D Y H S E L G D L G D I H D D D
H G V V N E S H R Y S W I N I F G D D S V L G R S I A I H Q
R D H L H K S A K I A C C V I G R G Q S H P E I V H R A K C
V V R P N T E S T G L H H H V S G S I T F E Q T P G G S T H
M T A D L K G F N V S E D L S H H R H G V Q L H E W G D M S
H G C H S L G R M Y H G H D D A H D P K R P G D L G D V I D
D S H G I V H S T R T F D H L N V E D L N A R S L V I M Q G
G H E V E S E R V A C C V I G R A (SEQ ID NO. 7)

or an active fragment thereof.

In yet a further aspect, the invention provides an isolated protein which is obtainable from the haemolymph of *Perna canaliculus* which has an apparent molecular weight of 75 kda determined by PAGE, or an active fragment thereof.

Conveniently, said protein or fragment has activity as:

(i) a serine protease inhibitor; or

(ii) a divalent cation binding agent.

The invention further provides a protein which is a functionally equivalent variant of a protein or fragment as defined above.

Still further, the invention provides a protein which is obtainable from a shellfish other than *Perna canaliculus* and which is a functionally equivalent variant of a protein or fragment as defined above.

In another aspect, the invention provides a polynucleotide encoding a protein or fragment as defined above.

The polynucleotide may comprise the nucleotide sequence of

5′ GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGA
CCACCACGATGATCACCATGACGACCATGATGATGATGATGAAACAATGCACT
ATGCCCAGTGTGAAATGGAACCAAACCCTCATATGGCTAGCAGCCTTCACCA
CCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCATGGAGCTGTTTAT
CTAGAACTTCATCTTGTCGGATTCAACACAAGTGAAGACCATGACGACCACCA
TCATGGACTTCATCTGCACATGCTTGGTGACATGTCAGCAGGTTGTGATTCTA
TTGGCGAACTGTACAATGCTCACCCAGAAAAACATGCTGACCCTGGTGACCT
CGGTGACCTGGTTGACGATGATAGGGGCGTGGTTAATGAAGTTCATCATTATG
CTTGGTTGGACATTGATGGTACAGCACCAAACACCGAAGCTCTCATTGGACA
CTCAATGACTATTTTACAAGGGAGTCACACCGATGCTGATACCCCAGCCAGTA
GAATCGCCTGTTGTGTTATTGGTCATGGAAAAGCTCGCCCAGAAACAGCAGC
TGCTCTACATCACGAGCTAGAGGAAGATAAAACTGAGCATTATGCCCATTGTG
ACGTAAGATCTAATACACACCAACCAAAGGCTCTTCATCATCATGTCCACGGA
ACCATCGATTTCAAACAAGTTGGTTATGGTGACCTTGAAGTGTCCTACCATTTA
GAGGGATTTAATGTAAGTGATGACCACAAAGATCATCTCCATGACGTACAGAT
CTACGCCAACGGTGACCTGACCAGTGGATGTGATAACCTCGGTGCTAAATAT
GATCCTCATGAAGATTACCACAGTGAGTTGGGTGATCTAGGAGATATTCACGA
TGATGACCATGGCGTTGTCAATGAAAGCCACAGATATTCCTGGATCAATATCT
TCGGTGATGACAGTGTCCTGGGACGTTCTATTGCCATTCACCAAAGAGACCAT
CTTCATAAAAGTGCCAAAATTGCCTGTTGTGTCATAGGACGTGGACAGAGCCA
TCCAGAAATTGTTCACAGAGCTAAATGTGTTGTCAGACCTAATACAGAATCTAC
TGGTTTACATCACCATGTCTCTGGTTCTATAACATTCGAACAGACCCCTGGAG

GATCAACACATATGACGGCTGATCTCAAAGGATTTAACGTTAGTGAGGACTTG
TCACATCATCGTCATGGTGTGCAGCTCCATGAATGGGGAGATATGTCCCATG
GCTGTCACTCCTTAGGCAGAATGTACCATGGTCATGATGATGCTCATGACCCC
AAAAGACCTGGTGACCTTGGTGATGTTATAGATGATTCCCATGGCATCGTTCA
TTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCT
TGTGATTATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGT
GTTATAGGACGGGCA (SEQ ID NO. 6)

or a variant thereof.

Still further, the invention provides a vector or construct which includes a polynucleotide as defined above.

In another aspect, the invention provides a composition which comprises a protein or fragment as defined above.

The composition may be a medicament, a food, a dietary supplement, (optionally including the protein associated with or bound to at least one divalent cation of dietary significance) or a bioremediation agent.

In still another aspect, the invention provides a process for obtaining a protein as defined above which comprises the step of centrifuging material containing *Perna canaliculus* haemolymph or an extract thereof and recovering the sedimented protein.

DESCRIPTION OF THE DRAWINGS

While the present invention is broadly as defined above, it also includes embodiments of which the following description provides examples. In particular, a better understanding of the present invention will be gained through reference to the accompanying drawings in which

Figure 1: Purification of pernin from mussel haemolymph

a) light-scattering band following centrifugation of *P. canaliculus* haemolymph in CsCl; haemolymph was first centrifuged at low speed to remove

haemocytes and then at high speed; the re-suspended pellet was then centrifuged in CsCl.

b) UV absorption profile (254 nm wavelength) from fractionation of the CsCl gradient; the light-scattering material in figure 1a appears as a peak.

c) protein composition in 1 ml fractions of a CsCl gradient following electrophoresis in a 12% polyacrylamide gel; the heavily stained (Coomassie) bands coincide with the position of the light-scattering and UV-absorbing regions of the gradient; the molecular weight was approximately 75 kDa as compared with polypeptide molecular weight standards (lane 6) (refer Figure 4a for standards). Lanes 1-5 and 7-9 contained samples from the CsCl gradient.

Figure 2: Virus-like particles observed by transmission electron microscopy of material in light scattering band in a CsCl gradient. Bar in micrograph represents 100 nm.

Figure 3: HPLC elution profile of pernin at 280 nm wavelength purified by CsCl gradient centrifugation..

Figure 4: SDS-PAGE profiles (12% gels) of aggregating protein species from *P. canaliculus* and other shellfish species

a) proteins extracted from whole shellfish and purified as described in Materials and Methods: lane 1: molecular weight standards (Bio-Rad, USA) :**pb** phosphorylase B, 97.4 kDa; **bsa** bovine serum albumin, 66 kDa; **ova** ovalbumin, 45 kDa; **ca** carbonic anhydrase, 31 kDa; lane 2: Greenshell™ mussel *P. canaliculus*; lane 3: blue mussel *Mytilis edulis*; lane 4: oyster *Crassostrea gigas*; lane 5: pipis *Paphies australis*.

b) PAGE analysis of human transferrin (Sigma, USA, MW *ca.* 80 kDa), a glycosylated protein, and pernin from *P. canaliculus* following treatment with endoglycosidase-F: lane 1: untreated transferrin; lane 2: transferrin treated

with glycosidase-F; lane 3: untreated pernin lane 4: pernin treated with glycosidase-F.

Figure 5: Activity of *P. canaliculus* haemolymph protein following centrifugation in a 30 kDa molecular weight exclusion filter for 10 min at 1000 *g* (Ultrafree-MC filter, 30,000 MW exclusion, Millipore, USA)

a) SDS-PAGE profile of haemolymph protein at various stages of purification. Lane 1: "crude" haemolymph (haemocytes removed); lane 2: resuspended pellet after ultracentrifugation of "crude" haemolymph for 80 min at 250,000 *g*; lane 3: pernin retentate; lane 4: filtrate (no proteins evident); lane 5: molecular weight markers, (refer Figure 4a); lanes 6,7: 10-fold dilutions of samples from lanes 2 and 3.

b) Anti-thrombin activity of 30,000 MW exclusion filter retentate and filtrate.

con+ = the standard 1/41 dilution of human plasma (i.e. standard anti-thrombin III activity);

con - thrombin with no added plasma (buffer control); **filtrate**: material passed through a 30,000 MW exclusion filter;

retentate: pernin protein retained by exclusion filter.

DESCRIPTION OF THE INVENTION

As broadly outlined above, in one aspect the present invention provides a novel protein. The protein of the invention has an apparent molecular weight of 75 kDa, calculated by polyacrylamide gel electrophoresis (PAGE). The molecular weight inferred from the gene sequence is approximately 55 kDa.

One specific protein of the invention was initially identified as an extract from the New Zealand green lipped mussel *P. canaliculus*. It is therefore obtainable by extraction directly from *P. canaliculus*.

This protein has the amino acid sequence of SEQ ID NO. 7.

The protein of the invention can include its entire native amino acid sequence or can include only parts of that sequence where such parts constitute fragments which remain biologically active (active fragments). Such activity will normally be as a serine protease inhibitor or a divalent cation binding agent but is not restricted to these activities.

The invention also includes within its scope functionally equivalent variants of the protein of SEQ ID NO. 7.

The phrase "functionally equivalent variants" recognises that it is possible to vary the amino acid of a protein while retaining substantially equivalent functionality. For example, a protein can be considered a functional equivalent of another protein for a specific function if the equivalent peptide is immunologically cross-reactive with and has at least substantially the same function as the original protein.

The functionally equivalent protein need not be the same size as the original. The equivalent can be, for example, a fragment of the protein, a fusion of the protein with another protein or carrier, or a fusion of a fragment with additional amino acids. It is also possible to substitute amino acids in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids normally held to be equivalent are:

(a) Ala, Ser, Thr, Pro, Gly;
(b) Asn, Asp, Glu, Gln;
(c) His, Arg, Lys;
(d) Met, Leu, Ile, Val; and
(e) Phe, Tyr, Trp.

Polypeptide sequences may be aligned, and percentage of identical amino acids in a specified region may be determined against another sequence, using computer algorithms that are publicly available. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. BLASTP software is available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The use of the BLAST family of algorithms, including BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication

of Altschul, Stephen F., *et al.* (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-34023.

The protein of the invention together with its active fragments and other variants may be generated by synthetic or recombinant means. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated by techniques well known to those of ordinary skill in the art. For example, such peptides may be synthesised using any of the commercially available solid-phase techniques such as the Merryfield solid phase synthesis method, where amino acids are sequentially added to a growing amino acid chain (see Merryfield, J. Am. Chem. Soc 85: 2146-2149 (1963)). Equipment for automative synthesis of peptides is commercially available from suppliers such as Perkin Elmer/Applied Biosystems, Inc. and may be operated according to the manufacturers instructions.

The protein, or a fragment or variant thereof, may also be produced recombinantly by inserting a polynucleotide (usually DNA) sequence that encodes the protein into an expression vector and expressing the protein in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule which encodes the recombinant protein. Suitable host cells includes procaryotes, yeasts and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeasts or a mammalian cell line such as COS or CHO, or an insect cell line, such as SF9, using a baculovirus expression vector. The DNA sequence expressed in this matter may encode the naturally occurring protein, fragments of the naturally occurring protein or variants thereof.

DNA sequences encoding the protein or fragments may be obtained by screening an appropriate *P. canaliculus* cDNA or genomic DNA library for DNA sequences that hybridise to degenerate oligonucleotides derived from partial amino acid sequences of the protein. Suitable degenerate oligonucleotides may be designed and synthesised by standard techniques and the screen may be performed as described, for example, in Maniatis *et al.* Molecular Cloning - A Laboratory Manual, Cold Spring Harbour Laboratories, Cold Spring Harbour, NY (1989). The polymerase

chain reaction (PCR) may be employed to isolate a nucleic acid probe from genomic DNA, a cDNA or genomic DNA library. The library screen may then be performed using the isolated probe.

Variants of the protein may be prepared using standard mutagenesis techniques such as oligonucleotide-directed site specific mutagenesis.

A specific polynucleotide of the invention has the nucleotide sequence of SEQ ID NO. 6 as follows:

5′ GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGA
CCACCACGATGATCACCATGACGACCATGATGATGATGATGAAACAATGCACT
ATGCCCAGTGTGAAATGGAACCAAACCCTCATATGGCTAGCAGCCTTCACCA
CCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCATGGAGCTGTTTAT
CTAGAACTTCATCTTGTCGGATTCAACACAAGTGAAGACCATGACGACCACCA
TCATGGACTTCATCTGCACATGCTTGGTGACATGTCAGCAGGTTGTGATTCTA
TTGGCGAACTGTACAATGCTCACCCAGAAAAACATGCTGACCCTGGTGACCT
CGGTGACCTGGTTGACGATGATAGGGGCGTGGTTAATGAAGTTCATCATTATG
CTTGGTTGGACATTGATGGTACAGCACCAAACACCGAAGCTCTCATTGGACA
CTCAATGACTATTTTACAAGGGAGTCACACCGATGCTGATACCCCAGCCAGTA
GAATCGCCTGTTGTGTTATTGGTCATGGAAAAGCTCGCCCAGAAACAGCAGC
TGCTCTACATCACGAGCTAGAGGAAGATAAAACTGAGCATTATGCCCATTGTG
ACGTAAGATCTAATACACACCAACCAAAGGCTCTTCATCATCATGTCCACGGA
ACCATCGATTTCAAACAAGTTGGTTATGGTGACCTTGAAGTGTCCTACCATTTA
GAGGGATTTAATGTAAGTGATGACCACAAAGATCATCTCCATGACGTACAGAT
CTACGCCAACGGTGACCTGACCAGTGGATGTGATAACCTCGGTGCTAAATAT
GATCCTCATGAAGATTACCACAGTGAGTTGGGTGATCTAGGAGATATTCACGA
TGATGACCATGGCGTTGTCAATGAAAGCCACAGATATTCCTGGATCAATATCT
TCGGTGATGACAGTGTCCTGGGACGTTCTATTGCCATTCACCAAAGAGACCAT
CTTCATAAAAGTGCCAAAATTGCCTGTTGTGTCATAGGACGTGGACAGAGCCA
TCCAGAAATTGTTCACAGAGCTAAATGTGTTGTCAGACCTAATACAGAATCTAC
TGGTTTACATCACCATGTCTCTGGTTCTATAACATTCGAACAGACCCCTGGAG
GATCAACACATATGACGGCTGATCTCAAAGGATTTAACGTTAGTGAGGACTTG
TCACATCATCGTCATGGTGTGCAGCTCCATGAATGGGGAGATATGTCCCATG
GCTGTCACTCCTTAGGCAGAATGTACCATGGTCATGATGATGCTCATGACCCC
AAAAGACCTGGTGACCTTGGTGATGTTATAGATGATTCCCATGGCATCGTTCA

TTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCT
TGTGATTATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGT
GTTATAGGACGGGCA.

A further polynucleotide has the sequence of SEQ ID NO. 8 as follows:

5'GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGA
CCACCACGATGATCACCATGACGACCATGATGATGATGATGAAACAATGCACT
ATGCCCAGTGTGAAATGGAACCAAACCCTCATATGGCTAGCAGCCTTCACCA
CCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCATGGAGCTGTTTAT
CTAGAACTTCATCTTGTCGGATTCAACACAAGTGAAGACCATGACGACCACCA
TCATGGACTTCATCTGCACATGCTTGGTGACATGTCAGCAGGTTGTGATTCTA
TTGGCGAACTGTACAATGCTCACCCAGAAAAACATGCTGACCCTGGTGACCT
CGGTGACCTGGTTGACGATGATAGGGGCGTGGTTAATGAAGTTCATCATTATG
CTTGGTTGGACATTGATGGTACAGCACCAAACACCGAAGCTCTCATTGGACA
CTCAATGACTATTTTACAAGGGAGTCACACCGATGCTGATACCCCAGCCAGTA
GAATCGCCTGTTGTGTTATTGGTCATGGAAAAGCTCGCCCAGAAACAGCAGC
TGCTCTACATCACGAGCTAGAGGAAGATAAAACTGAGCATTATGCCCATTGTG
ACGTAAGATCTAATACACACCAACCAAAGGCTCTTCATCATCATGTCCACGGA
ACCATCGATTTCAAACAAGTTGGTTATGGTGACCTTGAAGTGTCCTACCATTTA
GAGGGATTTAATGTAAGTGATGACCACAAAGATCATCTCCATGACGTACAGAT
CTACGCCAACGGTGACCTGACCAGTGGATGTGATAACCTCGGTGCTAAATAT
GATCCTCATGAAGATTACCACAGTGAGTTGGGTGATCTAGGAGATATTCACGA
TGATGACCATGGCGTTGTCAATGAAAGCCACAGATATTCCTGGATCAATATCT
TCGGTGATGACAGTGTCCTGGGACGTTCTATTGCCATTCACCAAAGAGACCAT
CTTCATAAAAGTGCCAAAATTGCCTGTTGTGTCATAGGACGTGGACAGAGCCA
TCCAGAAATTGTTCACAGAGCTAAATGTGTTGTCAGACCTAATACAGAATCTAC
TGGTTTACATCACCATGTCTCTGGTTCTATAACATTCGAACAGACCCCTGGAG
GATCAACACATATGACGGCTGATCTCAAAGGATTTAACGTTAGTGAGGACTTG
TCACATCATCGTCATGGTGTGCAGCTCCATGAATGGGGAGATATGTCCCATG
GCTGTCACTCCTTAGGCAGAATGTACCATGGTCATGATGATGCTCATGACCCC
AAAAGACCTGGTGACCTTGGTGATGTTATAGATGATTCCCATGGCATCGTTCA
TTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCT
TGTGATTATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGT
GTTATAGGACGGGCA**TGA**ATAACCTCACTAGAGTGACTTTGTCTAACATGACA

ATTAACAATTGTATAACTTCGCTAAAA**AATAAAA**CAATGACACAATGNAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAA3'

with TGA being the opal stop codon and AATAAA the polyadenylation signal.

Variants or homologues of the above polynucleotide sequences also form part of the present invention. Polynucleotide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The BLASTN software is available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F, *et al* (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu.pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in the W R Pearson and D.J. Lipman, "Improved Tools for Biological Sequence Analysis," *Proc. Natl. Acad. Sci. USA 85*:2444-2448 (1988) and W.R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," *Methods in Enzymology 183*:63-98 (1990).

All sequences identified as above qualify as "variants" as that term is used herein.

Variant polynucleotide sequences will generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1%

SDS at 65°C. Such hybridizable sequences include those which code for the equivalent protein from sources (such as shellfish) other than *P. canaliculus*.

While the above synthetic or recombinant approaches can be taken to produce the protein of the invention, it is however practicable (and indeed presently preferred) to obtain the protein by isolation from *P. canaliculus*. This reflects the applicants' finding that the protein is the dominant protein of the haemolymph of *P. canaliculus* and also that the protein is self-aggregating. It can therefore be isolated in commercially significant quantities direct from the mussel itself. For example, approximately 2 mg of the protein can be obtained per ml of haemolymph.

Once obtained, the protein is readily purified if desired. This will generally involve centrifugation in which the self-aggregating nature of the protein is important. Other approaches to purification (eg. chromatography) can however also be followed.

Furthermore, if viewed as desirable, additional purification steps can be employed using approaches which are standard in this art. These approaches are fully able to deliver a highly pure preparation of the protein.

Once obtained, the protein and/or its active fragments can be formulated into a composition. The composition can be, for example, a therapeutic composition for application as a pharmaceutical, or can be a health or dietary supplement. Again, standard approaches can be taken in formulating such compositions.

Still further, the composition can be a food in which the protein and/or its active fragments are included. This can occur by adding the protein to a pre-prepared foodstuff, or incorporating the protein into a step of the manufacturing process for the food.

The invention will now be described more fully in the following experimental section which is provided for illustrative purposes only.

EXPERIMENTAL

Section 1

A. Materials and Methods

A.1 Shellfish: *Perna canaliculus* (the New Zealand green-lipped mussel; the Greenshell™ mussel) were obtained at retail supermarket outlets or from mussel farmers directly; other shellfish species were obtained from retail outlets except for the blue mussel *Mytilis edulis* which was supplied by Sanford's Fisheries (Havelock, New Zealand).

A.2 Extracts: Mussel extracts were prepared by homogenising whole, shucked mussels (up to 120 mm length) in a commercial food processor with the addition of 0.02 M sodium phosphate buffer, pH 7.2. Dichloromethane (1/2 volume) was mixed with the aqueous extract, centrifuged at low speed (6000 rpm, GSA rotor, Sorvall RC-5B centrifuge at 4 °C). Polyethylene glycol (PEG) (MW 6000) was added to the aqueous phase to a final concentration of 10% (w/v) and NaCl to 0.5 M and stirred at 4-6 °C overnight. Following low speed centrifugation the PEG-precipitate was resuspended in approximately 1/10 volume of sodium phosphate buffer. After another cycle of low-speed centrifugation the supernatant was centrifuged at high speed (50,000 rpm in a Beckman 60Ti rotor at 4 °C for 60-80 minutes). The resultant pellet was resuspended in a small volume of phosphate buffer and clarified by low speed centrifugation.

A.3 Polyacrylamide gel electrophoresis: 12% polyacrylamide gels (8 x10 cm; 1 mm thick) were cast using a prepared stock solution according to the manufacturer's instructions (40% acrylamide/bis solution 37.5:1, Bio-Rad, USA); commercially available 12% gels (Bio-Rad, USA) were also used. Samples (10 µl) were applied to lanes and the gels run at 160 V using a standard Tris/Glycine/SDS buffer (Bio-Rad, catalogue 161-0732) until the bromphenol blue marker reached the bottom of the gel. Gels were stained with BM Fast Stain Coomassie® (Boehringer Mannheim, Germany) and destained as per the manufacturer's instructions.

A.4 **Glycosylation test:** Samples were treated with N-glycosidase F (PNGase F from *Flavobacterium meningosepticum*; Boehringer Mannheim Biochemica, Germany) according to the manufacturer's directions. Treated and untreated samples were run in a standard 12% polyacrylamide gel.

A.5 **Isopycnic gradients:** CsCl (Boehringer Mannhein, Germany) solutions were prepared in 0.1 M sodium phosphate buffer, pH 7.2 and filtered through a 0.22 μm membrane (Acrodisc, Gelman Sciences, USA) to clarify. Two step gradients (1.25 g/cc top layer containing the sample and 1.45 g/cc bottom layer) were prepared as described by Scotti (1985) and centrifuged for approximately 17 hours at 20 °C in a Beckman 70Ti rotor at 30,000 rpm. The resultant gradient was fractionated by inserting a 100 μl glass capillary tube into the gradient and slowly pumping out the contents. UV absorbance was monitored by passing through a Uvicord spectrophotometer (LKB Produkter, Sweden). Fractions were collected and the refractive indices measured using an Abbé refractometer (Bellingham and Stanley, UK) and the density estimated using regression equations according to the method of Scotti (1985).

A.6 **Porous glass chromatography:** Controlled pore glass (CPG 240-80, Sigma Chemical Co., USA) was treated according to the suppliers directions. A 1 cm x 100 cm column (Bio-Rad, USA) was prepared. Samples (1-2 ml) were loaded onto the column and eluted with 0.1 M sodium phosphate buffer, pH 7.2, through a Uvicord spectrophotometer, fractions being collected at regular intervals.

A.7 **Estimation of protein concentration:** Concentrations were estimated using a bovine serum albumin standard (Blot Qualified BSA, Promega, USA) by UV absorption according to the method of Layne (1957) using the equation: mg/ml protein = $1.55*A_{280} - 0.76*A_{260}$. Alternatively, concentration was estimated by the Bradford reaction using reagent supplied by Bio-Rad (USA) at a wavelength of 620 nm..

A.8 High performance liquid chromatography: Reversed-phase HPLC was performed on an HP 1050 Ti-series HPLC (Hewlett Packard, USA) fitted with an analytical 300 Å Vydac C-18 column, 25 cm x 4.6 mm i.d.. The 10 µl sample in water was eluted with a 0-100% acetonitrile in water (v/v) gradient over 60 min and the absorption at 218 and 280 nm was recorded.

B. Results

A light-scattering band was seen after centrifugation of extracts of whole Greenshell™ mussels in CsCl gradients **(Figures 1a and 1b)**. The density of this band was estimated at 1.368 g/cc. A minor band was sometimes observed at approximately 1.390 g/cc. If rebanded in CsCl the 1.390 band yielded two bands - one at 1.390 g/cc and a second at 1.368 g/cc. SDS-PAGE analysis of fractions of either density gave similar polypeptide profiles with a single major band. The molecular weight of the protein by PAGE was estimated as 75,000 (75 kDa) **(Figure 1c)**. Several minor bands of higher molecular weight and an additional minor band of 45 kDa were also seen. The main band (called pernin) at 75 kDa was always at great excess compared to the minor bands. When material from the light-scattering material from CsCl gradients were examined by electron microscopy, particles resembling those of "empty" small RNA viruses were seen **(Figure 2)**. However a UV wavelength scan (data not shown) indicated that little, if any, nucleic acid was present and that the particles were mainly composed of protein. HPLC showed the CsCl band to be composed almost solely of a single species of protein **(Figure 3)**. Since HPLC indicated a high degree of purity, the higher molecular weight polypeptides are presumed to be multimers of pernin. It is likely that the minor, lower molecular weight band is degraded pernin.

Chromatography, on a CPG 240-80 column, of semi-purified extracts, or of material banded in CsCl, showed that the majority of pernin was eluted in the exclusion volume using low molarity phosphate or Tris buffer as the eluent. In contrast, a protein of similar size, bovine serum albumin (68 kDa), was included in the column matrix. It appears, therefore, that pernin does aggregate into large, particle-like structures under certain conditions as suspected from the particles seen in **Figure 2**. HPLC confirmed that pernin from *P. canaliculus* obtained by CPG chromatography was highly purified. Aggregating protein species were also detected in extracts of

other shellfish: the blue mussel *Mytilis edulis*, the oyster *Crassostrea gigas*, and New Zealand pipis *Paphies australis* but not in scallops *Pecten novaezealandiae*. These polypeptides were lower in molecular weight than pernin **(Figure 4a)**. The pernin from *P. canaliculus* is N-glycosylated as shown by a reduction in molecular weight when treated with endoglycosidase-F before PAGE **(Figure 4b)**.

The yield of pernin from whole mussel extractions averaged about 200 μg/mussel. Improved yields of pernin were obtained by extracting haemolymph directly from live *P. canaliculus*. A small notch was made in the shell using a triangular file and a 30 gauge needle inserted into the posterior adductor muscle. From 1 to 5 ml of haemolymph can be withdrawn easily. The haemolymph was spun at low speed (≈1000 *g*) to remove haemocytes and the resulting supernatant processed by ultracentrifugation, for example at 250,000 *g* for 40 minutes, followed by either CPG chromatography eluting with 0.1 M sodium phosphate buffer, pH 7.2, or isopycnic banding in CsCl in phosphate buffer. The pernin obtained in this way appeared no different than that purified from whole mussels and had the advantage of a 30-fold average increase in yield from each mussel. Haemolymph contained around 2 mg/ml (average ≈5-6 mg/mussel) of pernin which is by far the most predominant polypeptide species (**Figure 5a**). The time to purify pernin was reduced from about 5 days to 1 day.

Microsequencing of the N-terminal region and internal fragments generated by chemical and enzymatic cleavage from purified pernin was performed and generated the following sequences of cleavage fragments:

(a) DGEQCNDGQN

(b) QGGHEVESERVACCVIGRA

(c) GQSHPEIVH

(d) YHGHDDA

(e) VVNEVHH.

These sequences code for amino acids as follows:

CODE:	
A	alanine
C	cystine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

The sequence data was then compared with amino acid sequences in searchable computer data bases. Some sequences were found to be of particular interest:

a) a 10 amino acid residue sequence from the N-terminus of pernin (sequence (a) above) showed <u>only</u> homology with an 8 base anti-thrombin protein sequence from terrestrial leeches (data from US Patent 5,455,181 Oct 3, 1995: sequence 10).

Perna canaliculus pernin	**2** GEQCNDGQ **9**
matching amino acids	***G+ CNDGQ***
leech anti-thrombin	**5** GQSCNDGQ **12**

identities: 6/8 (75%) positives: 7/8 (87%);
"+" indicates an equivalent amino acid;
the bolded numerals indicate amino acid position

b) An internal cleavage product (sequence (b) above) was shown to be have homology to the Cu-Zn class of proteins known as "SODs" (superoxide dismutases).

Each of fragments (a) to (e) are part of the larger pernin amino acid sequence:

1	**DGEQCNDGQN**	KDDHHDDHHD	DHHDDHDDDD	ETMHYAQCEM	EPNPHMASSL
5	HHHVHGSIEL	SQKGHGAVYL	ELHLVGFNTS	EDHDDHHHGL	HLHMLGDMSA
0	GCDSIGELYN	AHPEKHADPG	DLGDLVDDDR	G**VVNEVHH**YA	WLDIDGTAPN
5	TEALIGHSMT	ILQGSHTDAD	TPASRIACCV	IGHGKARPET	AAALHHELEE
20	DKTEHYAHCD	VRSNTHQPKA	LHHHVHGTID	FKQVGYGDLE	VSYHLEGFNV
25	SDDHKDHLHD	VQIYANGDLT	SGCDNLGAKY	DPHEDYHSEL	GDLGDIHDDD
30	HGVVNESHRY	SWINIFGDDS	VLGRSIAIHQ	RDHLHKSAKI	ACCVIGR**GQS**
35	**HPEIVH**RAKC	VVRPNTESTG	LHHHVSGSIT	FEQTPGGSTH	MTADLKGFNV
40	SEDLSHHRHG	VQLHEWGDMS	HGCHSLGRM**Y**	**HGHDDA**HDPK	RPGDLGDVID
45	DSHGIVHSTR	TFDHLNVEDL	NARSLVIM**QG**	**GHEVESERVA**	**CCVIGRA**

(Bold characters indicate directly sequenced fragments (a) to (e)).

Section 2

Anti-thrombin Activity

The possibility that pernin could function as an anti-thrombin agent was examined in a kinetic assay for thrombin inhibition.

Thrombin inhibition assay

Kinetic assays were done using an Accucolor™ Antithrombin III kit (catalogue no. CRS105, Sigma Diagnostics, USA) with the reagents prepared according to the supplier's directions. Standard plasma was supplied by Instrumentation Laboratories (Italy) and used at the recommended dilution of 1/41. Samples of purified mussel protein in water were diluted 9/10 by adding 10X Sigma sample buffer. Heparin was purchased from Instrumentation Laboratories. Thrombin activity was estimated colorimetrically at 405 nm using a chromogenic substrate (H-D-HHT-L-Ala-L-Arg-pNa.2AcOH, catalogue no. A 8058, Sigma, USA) and a Multiskan Biochromatic plate reader (Labsystems, Finland)

This verified that pernin had inhibitory activity. When a purified preparation of pernin was centrifuged through a 30,000 MW exclusion filter **(Figure 5a)**, all the anti-thrombin activity was in the retentate and no detectable activity was present in

the filtrate **(Figure 5b).** The standard serum was diluted 1/41 as recommended for this assay system; the pernin concentration was not determined directly but was in the 1 mg/ml range. From this kinetic data pernin inhibition was estimated to be about 50% of the level of human plasma (approximately 1 mg/ml pernin diluted 9/10 compared with the 1/41 plasma dilution in the standard ATIII assay system). Heparin, a co-factor required for ATIII inhibition of thrombin, was not required for inhibitory action by pernin.

Metal Binding Activity

Hi Trap® Chelating affinity columns (Amersham Pharmacia Biotech, 1ml size) were prepared according to the manufacturer's instructions. The columns were then charged with either 0.1M cupric chloride or zinc chloride before equilibrating in a buffer (0.050M sodium phosphate and 0.5M sodium chloride containing 0.5mM imidazole, pH 7.0). Protein samples purified using CsCl centrifugation were suspended in this buffer and applied to the column using a chromatographic system (Econo System, Bio-Rad Laboratories, USA). Following washing of the column for 5 mins with buffer during which no protein appeared in the eluate, a linear gradient over 20 min at 1 ml/min was used to develop the column using buffer with the imidazole concentration at 100mM from 0-100%. The protein eluted into the gradient being retained longer on the copper chelation column than the zinc. The absorption of the eluate was monitored at 254nM.

Divalent metal ion content of the CsCl purified protein was determined by dissolving the protein in water at 10 mg/ml and analysing metal content by both atomic absorption and plasma emission spectrometry by comparison with a water blank. There was no significant divalent cation content in the protein purified by this method. However, purification by other methods not employing chaotropic agents like CsCl, the high content of histidine coupled with acidic amino acid residues and the likely origin of this protein from a SOD precursor, points to pernin having endogenous metal ions as part of its native structure.

Section 3

Gene Sequencing Method

A suite of non-specific primers called pUZ5 was synthesised by Gibco-BRL for the initial sequencing based on the N-terminal sequence of pernin. The general formula was:

GAY GGN GAR CAR TGY AAY GAY GGN CAR AA

Where Y represents a pyrimidine base, R represents a purine base and N represents any one of the four nucleotide bases. Sequencing was done, initially using pUZ5 and an oligo-dT based "bottom stand" primer from PCR amplified cDNA. Sequencing was done by dye-termination cycle sequencing using "BigDye" prism technology (Applied Biosystems Incorporated, USA) according to their instructions. Products were resolved on an ABI 377 automated sequencer. Following the initial sequencing of approximately 500 base pairs pernin-specific primers were constructed and used to complete the sequencing of the pernin gene.

This provided the following:

GAYGGGGAGCAGTGTAACGATGGGCAGAACAAAGATGACCACCATGACGACCACCACGATGATCA
CCATGACGACCATGATGATGATGATGAAACAATGCACTATGCCCAGTGTGAAATGGAACCAAACC
CTCATATGGCTAGCAGCCTTCACCACCATGTCCATGGCAGCATAGAGTTGTCACAGAAGGGTCAT
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GTTCATTCAACTAGAACCTTTGATCATCTTAATGTTGAAGATCTTAACGCACGTTCCCTTGTGAT
TATGCAGGGCGGACATGAGGTCGAGAGTGAGAGGGTTGCTTGCTGTGTTATAGGACGGGCA**TGA**A
TAACCTCACTAGAGTGACTTTGTCTAACATGACAATTAACAATTGTATAACTTCGCTAAAA**AATA**
AAACAATGACACAATGNAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA.

Discussion

The present invention is a novel protein obtainable from *Perna canaliculus*, the New Zealand green-lipped (Greenshell™) mussel. The protein appears to be able to self-aggregate in structures resembling small virus like particles (VLPs) approximately 25 nm in diameter but lacking any nucleic acid. The protein was found in extracts of whole mussels and appears to be the predominant protein in haemolymph. The molecular weight of the protein was estimated to be 75 kDa by PAGE and inferred to be 55 kDa from its polynucleotide encoding sequence but, because of its ability to aggregate, the protein can be sedimented by ultracentrifugation in a short time (e.g. 40 minutes at 250,000 *g*) whereas the monomeric protein would not. Each ml of haemolymph yields, on the average, about 2 mg of pernin. Haemolymph is easily obtained by withdrawing fluid from the posterior adductor muscle of the shellfish which can yield up to 5 ml without obvious harm; it is not necessary to kill the mussel. The haemolymph obtained not only contains high levels of pernin but is quite free of contaminating materials, particularly compared with whole mussel extracts, so purification of pernin is simple. For highly pure preparations of pernin, ultracentrifugation is followed by isopycnic banding in a suitable density gradient medium such as CsCl.

The sequence of the N-terminus of pernin suggested that the protein might have anti-thrombin activity. This was demonstrated in kinetic assays on purified pernin. Since thrombin is a serine protease, pernin also acts as a serine protease inhibitor.

Comparison of the sequences obtained from several cleavage fragments against amino acid sequences in a computer database suggest that in addition to the anti-thrombin activity of pernin, the protein also possesses other activities. One of these is the ability to bind divalent cations such as Zn^{2+} and Cu^{2+}.

INDUSTRIAL APPLICATION

The preferred protein of the invention, pernin, has a number of utilities.

Because of its anti-thrombin activity pernin is potentially useful as an anti-coagulant agent. Thrombin normally acts as a protease which converts fibrinogen in the blood to fibrin. Blood coagulation is counteracted by inhibitors, normally anti-thrombin III (ATIII); pernin has also been shown to inhibit thrombin activity in an ATIII assay system. In contrast to ATIII, whose action is accelerated by the presence of heparin (a sulphated mucopolysaccharide) pernin does not require heparin as a co-factor.

The pernin protein from *P. canaliculus* thus has value as a pharmaceutical. Since it is active as an anticoagulant in its native state it may also be useful as a natural therapeutic agent or health supplement. It is readily obtained as a natural product in high concentrations from mussel haemolymph. To obtain a highly pure preparation it is necessary only to remove haemocytes by centrifugation (or any other suitable method) followed by either ultracentrifugation (since pernin forms aggregates which readily sediment) and resuspension, isopycnic banding in a suitable medium such as CsCl, exclusion filtration through a suitable membrane which retains pernin, or chromatography through a medium such as controlled pore glass of suitable porosity. The result is a highly pure preparation of pernin.

The mussel *P. canaliculus* produces large amounts of the protein naturally, with little cost or effort involved in production, processing or purification.

A further utility of the protein arises from the fact that pernin can be stripped of divalent cations (for example by CsCl isopycnic banding, or pH variation). This allows for the addition of divalent cations of choice (such as Mg^{++}, Cd^{++}, Zn^{++} or Ca^{++}) to the metal stripped pernin. Such a protein, with a modified and pre-selected divalent cation loading, has application in the food and nutraceutical industries.

The ability to bind divalent metal cations also gives rise to applications of the protein in bioremediation and/or cation recovery processes. The divalent cations

can be present as contaminants or pollutants in, for example, a solution, and the solution passed by a substrate to which the protein is bound so that the cations are extracted.

Yet a further utility arises from the fact that the protein is "self-aggregating", and can form into structures resembling empty virus-like particles of approximately 25 nm in diameter. These empty virus-like particles are able to sequester other molecules inside them, with the consequent ability to function as delivery vehicles for those other molecules. Examples of molecules able to be delivered in this manner include pharmaceutically active compounds.

Those persons skilled in the art will understand that the above description is provided by way of illustration only and that the invention is limited only by the appended claims.

REFERENCES

Layne, E. (1957). Spectrophotometric and turbidometric methods for measuring proteins, *Methods in Enzymology* **III**, 447.

Scotti, P.D. (1985). The estimation of virus density in isopycnic cesium chloride gradients. *Journal of Virological Methods* **12**, 149.

CLAIMS:

1. An isolated protein which has a molecular weight of about 55 kDa and an amino acid sequence which includes one or more of the following:

(a) SEQ ID NO. 1

(b) SEQ ID NO. 2

(c) SEQ ID NO. 3

(d) SEQ ID NO. 4

(e) SEQ ID NO. 5

or an active fragment thereof.

2. An isolated protein which comprises the amino acid sequence of SEQ ID NO. 7, or an active fragment thereof.

3. An isolated protein which is obtainable from the haemolymph of *Perna canaliculus* which has an apparent molecular weight of 75 kDa determined by PAGE, or an active fragment thereof.

4. A protein or fragment as claimed in any one of claims 1 to 3 which has activity as:

(i) a serine protease inhibitor; or

(ii) a divalent cation binding agent.

5. A protein or fragment as claimed in claim 4 which has activity as a serine protease inhibitor.

6. A protein or fragment as claimed in claim 4 which has activity as a divalent cation binding agent.

7. A protein which is a functionally equivalent variant of a protein or fragment as claimed in 5 or 6.

8. A protein which is obtainable from a shellfish other than *Perna canaliculus* and which is a functionally equivalent homologue of a protein or fragment as claimed in claim 5 or 6.

9. A polynucleotide encoding a protein or fragment as claimed in any one of claims 1 to 8.

10. A polynucleotide as claimed in claim 9 which comprises the nucleotide sequence of SEQ ID NO. 6 or a variant thereof.

11. A polynucleotide which has the nucleotide sequence of SEQ ID NO. 8.

12. A vector which includes a polynucleotide as claimed in any one of claims 9 to 11.

13. A host cell which expresses a polynucleotide as claimed in any one of claims 9 to 11.

14. A composition which comprises a protein or fragment as claimed in any one of claims 1 to 8.

15. A composition as claimed in claim 14 which is a medicament.

16. A composition as claimed in claim 14 which is a food.

17. A composition as claimed in claim 14 which is a dietary supplement.

18. A dietary supplement as claimed in claim 17 in which said protein or fragment is associated with or bound to at least one divalent cation of dietary significance.

19. A dietary supplement as claimed in claim 18 wherein said divalent metal cation is calcium, magnesium or zinc.

20. A composition as claimed in claim 14 which is a bioremediation agent.

21. A process for obtaining a protein as claimed in claim 3 which comprises the step of centrifuging material containing *Perna canaliculus* haemolymph or an extract thereof and recovering the sedimented protein.

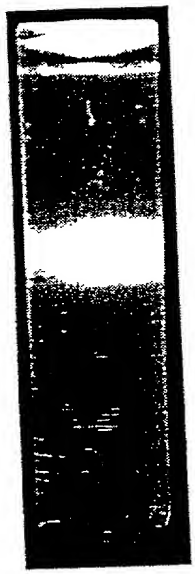
22. A process as claimed in claim 21 wherein said centrifuging step is ultracentrifugation.

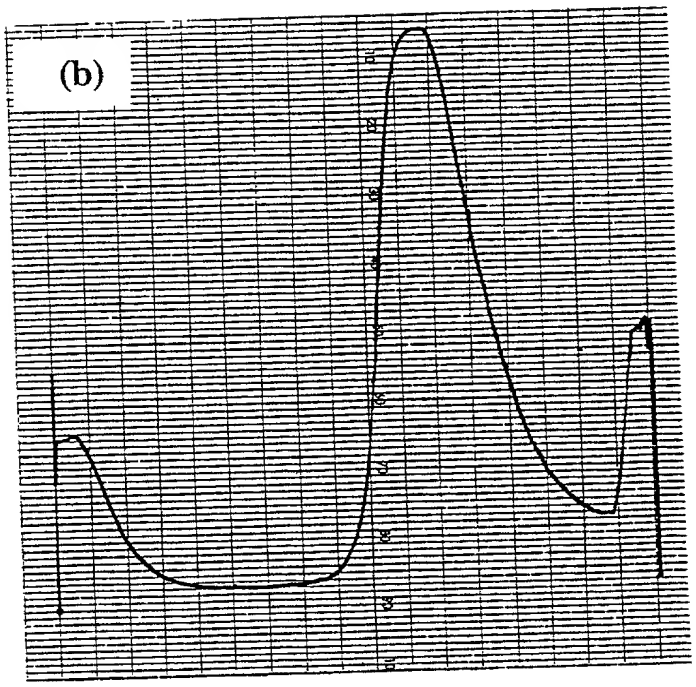
23. A process as claimed in claim 22 wherein said ultracentrifugation is performed for about 40 minutes at about 250,000g.

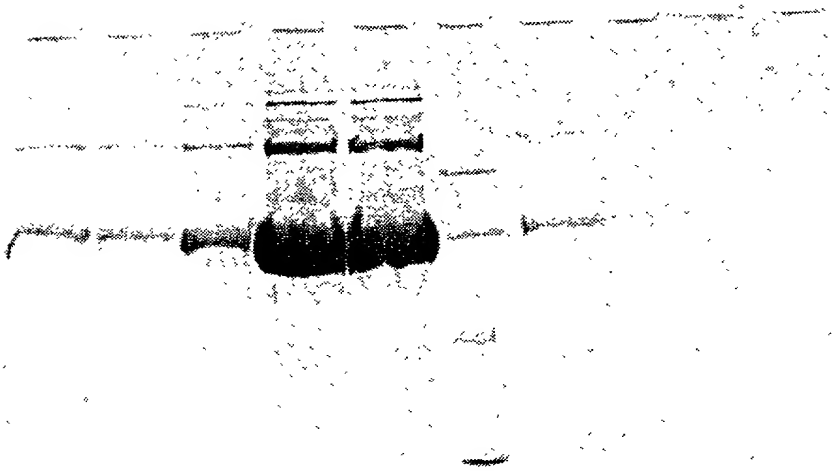
24. A process as claimed in any one of claims 21 to 23 which includes the preliminary step of extracting haemolymph from *Perna canaliculus*.

Figure 1

(a)







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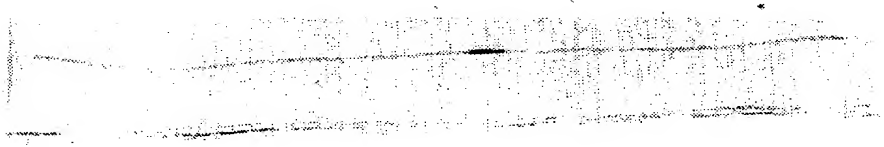

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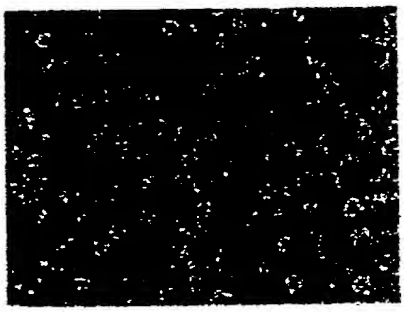

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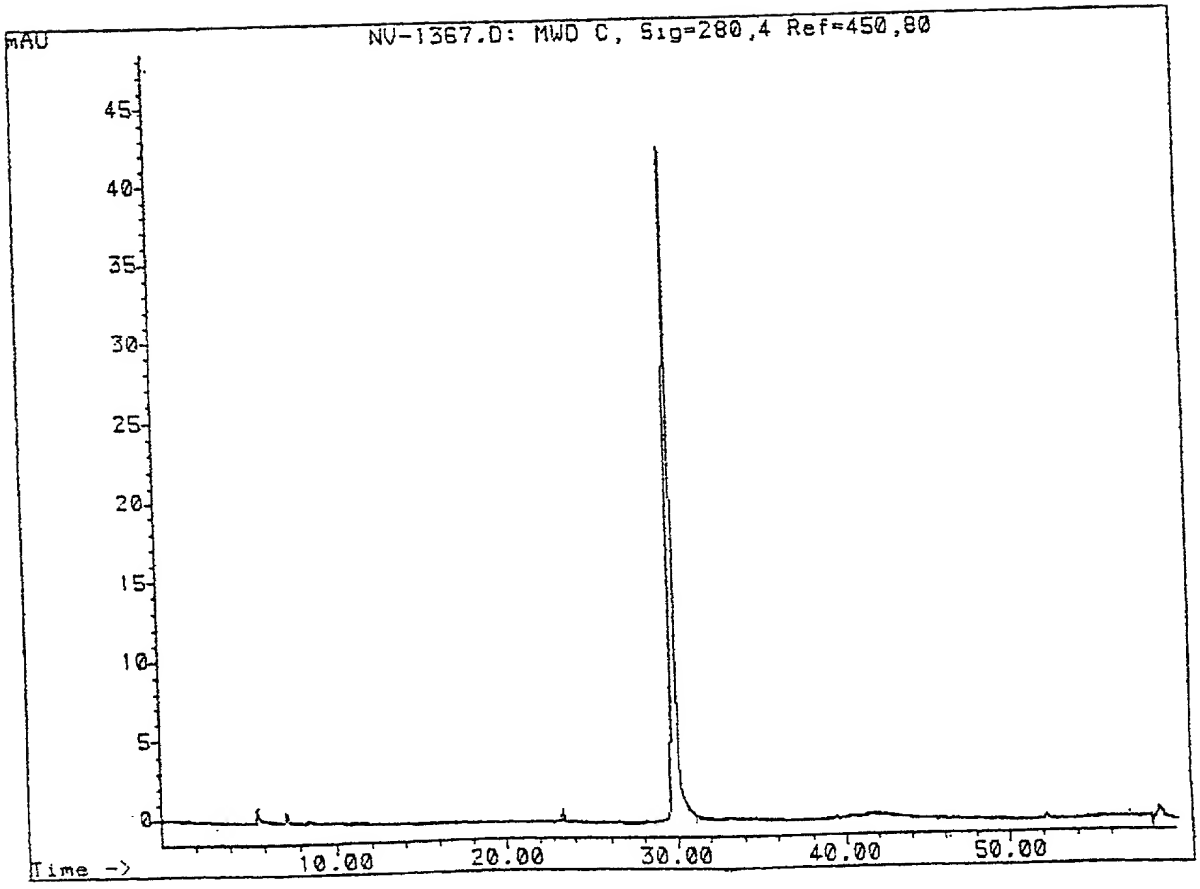

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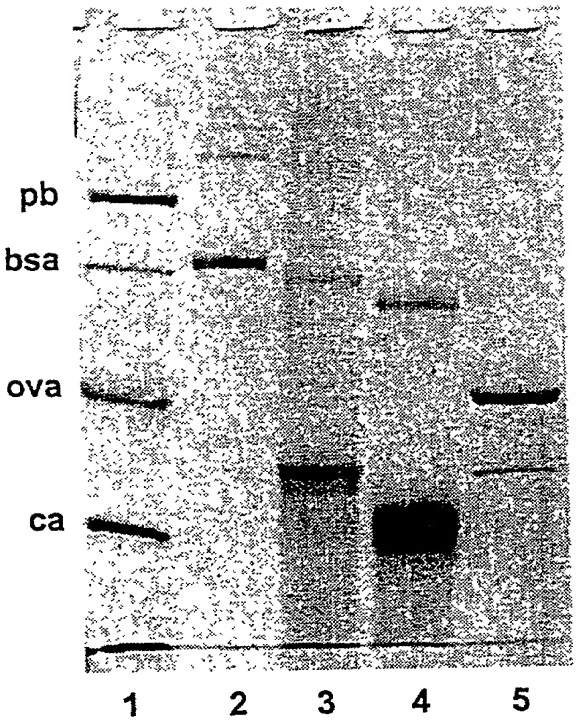

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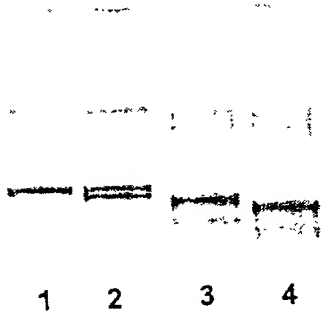

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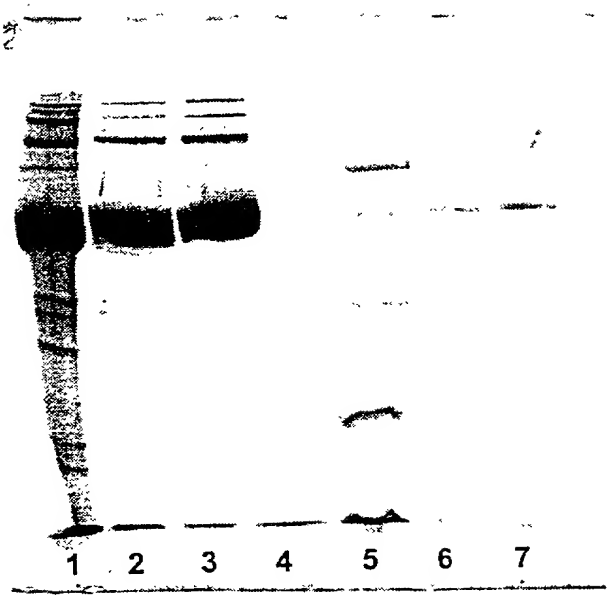
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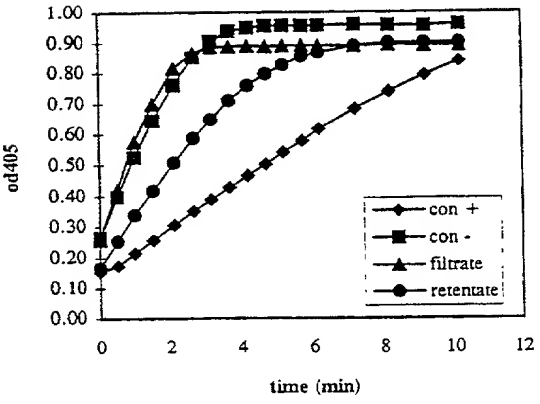
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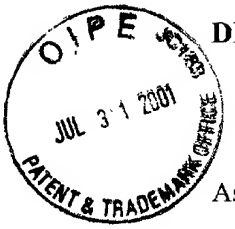

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Under 37 CFR § 1.63; includes reference to PCT International Applications)

FROMMER LAWRENCE & HAUG LLP

File No.: 514274-2001

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED: SERINE PROTEASE INHIBITOR, the specification of which ☐ is attached hereto ☐ was filed on as ☐ United States ☐ PCT Application No. , with amendments through (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No
New Zealand	333568	23 December 1998	X	☐
New Zealand	336906	23 July 1999	X	☐

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States application listed below:

_____ (Application Number) (Filing Date)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)
	23 December 1999	PCT/NZ99/00227	Pending

I hereby appoint , Registration No. , and Frommer Lawrence & Haug LLP, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications

DECLARATION FOR PATENT APPLICATION
AND POWER OF ATTORNEY (Under 37 CFR § 1.63)

FLH Docket No.

thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and specify that all communications about the application are to be directed to the following correspondence address:

, Esq.
c/o FROMMER LAWRENCE & HAUG LLP
745 Fifth Avenue
New York, NY 10151

Direct all telephone calls to: (212) 588-0800
to the attention of:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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1-00
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Citizenship: New Zealand

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2-00
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3-00
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Citizenship: New Zealand

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4-00
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Citizenship: New Zealand

Post Office Address(es) of inventors [if different from residence]:

NOTE: In order to qualify for reduced fees available to Small Entities, each inventor and any other individual or entity having rights to the invention must also sign an appropriate separate "Verified Statement (Declaration) Claiming [or Supporting a Claim by Another for] Small Entity Status" form [e.g. for Independent Inventor, Small Business Concern, Nonprofit Organization, Individual Non-Inventor].

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gaa gac cat gac gac cac cat cat gga ctt cat ctg cac atg ctt ggt 288
Glu Asp His Asp Asp His His His Gly Leu His Leu His Met Leu Gly
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Asp Met Ser Ala Gly Cys Asp Ser Ile Gly Glu Leu Tyr Asn Ala His
100 105 110

cca gaa aaa cat gct gac cct ggt gac ctc ggt gac ctg gtt gac gat 384
Pro Glu Lys His Ala Asp Pro Gly Asp Leu Gly Asp Leu Val Asp Asp
115 120 125

gat agg ggc gtg gtt aat gaa gtt cat cat tat gct tgg ttg gac att 432
Asp Arg Gly Val Val Asn Glu Val His His Tyr Ala Trp Leu Asp Ile
130 135 140

gat ggt aca gca cca aac acc gaa gct ctc att gga cac tca atg act 480
Asp Gly Thr Ala Pro Asn Thr Glu Ala Leu Ile Gly His Ser Met Thr
145 150 155 160

att tta caa ggg agt cac acc gat gct gat acc cca gcc agt aga atc 528
Ile Leu Gln Gly Ser His Thr Asp Ala Asp Thr Pro Ala Ser Arg Ile
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Ala Cys Cys Val Ile Gly His Gly Lys Ala Arg Pro Glu Thr Ala Ala
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gct cta cat cac gag cta gag gaa gat aaa act gag cat tat gcc cat 624
Ala Leu His His Glu Leu Glu Glu Asp Lys Thr Glu His Tyr Ala His
195 200 205

tgt gac gta aga tct aat aca cac caa cca aag gct ctt cat cat cat 672
Cys Asp Val Arg Ser Asn Thr His Gln Pro Lys Ala Leu His His His
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Val His Gly Thr Ile Asp Phe Lys Gln Val Gly Tyr Gly Asp Leu Glu

225 230 235 240

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Val Ser Tyr His Leu Glu Gly Phe Asn Val Ser Asp Asp His Lys Asp
245 250 255

cat ctc cat gac gta cag atc tac gcc aac ggt gac ctg acc agt gga 816
His Leu His Asp Val Gln Ile Tyr Ala Asn Gly Asp Leu Thr Ser Gly
260 265 270

tgt gat aac ctc ggt gct aaa tat gat cct cat gaa gat tac cac agt 864
Cys Asp Asn Leu Gly Ala Lys Tyr Asp Pro His Glu Asp Tyr His Ser
275 280 285

gag ttg ggt gat cta gga gat att cac gat gat gac cat ggc gtt gtc 912
Glu Leu Gly Asp Leu Gly Asp Ile His Asp Asp Asp His Gly Val Val
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Asn Glu Ser His Arg Tyr Ser Trp Ile Asn Ile Phe Gly Asp Asp Ser
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Val Leu Gly Arg Ser Ile Ala Ile His Gln Arg Asp His Leu His Lys
325 330 335

agt gcc aaa att gcc tgt tgt gtc ata gga cgt gga cag agc cat cca 1056
Ser Ala Lys Ile Ala Cys Cys Val Ile Gly Arg Gly Gln Ser His Pro
340 345 350

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Glu Ile Val His Arg Ala Lys Cys Val Val Arg Pro Asn Thr Glu Ser
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Thr Gly Leu His His His Val Ser Gly Ser Ile Thr Phe Glu Gln Thr
370 375 380

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Pro Gly Gly Ser Thr His Met Thr Ala Asp Leu Lys Gly Phe Asn Val
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Ser Glu Asp Leu Ser His His Arg His Gly Val Gln Leu His Glu Trp
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Gly Asp Met Ser His Gly Cys His Ser Leu Gly Arg Met Tyr His Gly

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Leu Asn Val Glu Asp Leu Asn Ala Arg Ser Leu Val Ile Met Gln Gly
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Ser Leu His His His Val His Gly Ser Ile Glu Leu Ser Gln Lys Gly
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His Gly Ala Val Tyr Leu Glu Leu His Leu Val Gly Phe Asn Thr Ser
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Glu Asp His Asp Asp His His His Gly Leu His Leu His Met Leu Gly
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Asp Met Ser Ala Gly Cys Asp Ser Ile Gly Glu Leu Tyr Asn Ala His
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Pro Glu Lys His Ala Asp Pro Gly Asp Leu Gly Asp Leu Val Asp Asp
115 120 125

Asp Arg Gly Val Val Asn Glu Val His His Tyr Ala Trp Leu Asp Ile
130 135 140

Asp Gly Thr Ala Pro Asn Thr Glu Ala Leu Ile Gly His Ser Met Thr
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Ile Leu Gln Gly Ser His Thr Asp Ala Asp Thr Pro Ala Ser Arg Ile
165 170 175

Ala Cys Cys Val Ile Gly His Gly Lys Ala Arg Pro Glu Thr Ala Ala
180 185 190

Ala Leu His His Glu Leu Glu Glu Asp Lys Thr Glu His Tyr Ala His
195 200 205

Cys Asp Val Arg Ser Asn Thr His Gln Pro Lys Ala Leu His His His
210 215 220

Val His Gly Thr Ile Asp Phe Lys Gln Val Gly Tyr Gly Asp Leu Glu
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Val Ser Tyr His Leu Glu Gly Phe Asn Val Ser Asp Asp His Lys Asp
245 250 255

His Leu His Asp Val Gln Ile Tyr Ala Asn Gly Asp Leu Thr Ser Gly
260 265 270

Cys Asp Asn Leu Gly Ala Lys Tyr Asp Pro His Glu Asp Tyr His Ser
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Glu Leu Gly Asp Leu Gly Asp Ile His Asp Asp Asp His Gly Val Val
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Asn Glu Ser His Arg Tyr Ser Trp Ile Asn Ile Phe Gly Asp Asp Ser
305 310 315 320

Val Leu Gly Arg Ser Ile Ala Ile His Gln Arg Asp His Leu His Lys
325 330 335

Ser Ala Lys Ile Ala Cys Cys Val Ile Gly Arg Gly Gln Ser His Pro
340 345 350

Glu Ile Val His Arg Ala Lys Cys Val Val Arg Pro Asn Thr Glu Ser
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Pro Gly Gly Ser Thr His Met Thr Ala Asp Leu Lys Gly Phe Asn Val
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Gly Asp Met Ser His Gly Cys His Ser Leu Gly Arg Met Tyr His Gly
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His Asp Asp Ala His Asp Pro Lys Arg Pro Gly Asp Leu Gly Asp Val
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Ile Asp Asp Ser His Gly Ile Val His Ser Thr Arg Thr Phe Asp His
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Leu Asn Val Glu Asp Leu Asn Ala Arg Ser Leu Val Ile Met Gln Gly
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